



Design of experiments and principal component analysis as approaches for enhancing performance of gas-diffusional air-breathing bilirubin oxidase cathode



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HIGHLIGHTS

- Two methods were used for the enhancement of gas-diffusional enzymatic electrodes.
- Based on the results from the DOE, an improved cathode was constructed.
- The current of the improved cathode was 2–5 times higher than the initial ones.
- Three major factors contributing to the cathode performance were identified.
- PCA is applied as an independent tool in order to support conclusions made by DOE.

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ABSTRACT

Two statistical methods, design of experiments (DOE) and principal component analysis (PCA) are employed to investigate and improve performance of air-breathing gas-diffusional enzymatic electrodes. DOE is utilized as a tool for systematic organization and evaluation of various factors affecting the performance of the composite system. Based on the results from the DOE, an improved cathode is constructed. The current density generated utilizing the improved cathode ($755 \pm 39 \mu\text{A cm}^{-2}$ at 0.3 V vs. Ag/AgCl) is 2–5 times higher than the highest current density previously achieved. Three major factors contributing to the cathode performance are identified: the amount of enzyme, the volume of phosphate buffer used to immobilize the enzyme, and the thickness of the gas-diffusion layer (GDL). PCA is applied as an independent confirmation tool to support conclusions made by DOE and to visualize the contribution of factors in individual cathode configurations.

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1. Introduction

With the increasing interest in the area of biofuel cells, the need for low-cost, highly efficient enzymatic cathodes has been observed [1–4]. Such cathodes include gas-diffusional air-breathing (GDAB) electrodes, which utilize enzymes to reduce oxygen to water. GDAB electrodes use oxygen as final electron acceptor, which is cheap, ubiquitous and easily accessible. Their main advantage over the

cathodes which reduce dissolved oxygen is that GDAB electrodes minimize losses due to O_2 diffusion through the liquid electrolyte, thus increasing the amount of accessible oxygen, providing it directly from the air by passive diffusion through the electrode. The values of diffusion coefficients of O_2 in water solutions and in the air are $2.10^{-5} \text{ cm}^2 \text{ s}^{-1}$ and $2.10^{-1} \text{ cm}^2 \text{ s}^{-1}$, respectively, and the amount of oxygen that can be dissolved in water is only $0.25 \text{ mmol dm}^{-3}$ [5]. Air-breathing gas-diffusional cathodes can improve the oxygen mass transfer due to the presence of high gas–solid–liquid boundary area, which makes them the preferable design toward higher current densities. The enhanced oxygen delivery and availability at the cathode leads to an increase in the electrode operational characteristics [6,7].

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In recent years several biocathodes, utilizing enzymes from the family of multi-copper oxidases (MCOs), were developed [3,5,7,8]. MCO is a group of enzymes (laccase, bilirubin oxidase, ascorbate oxidase, etc.) that can reduce oxygen during the oxidation of their natural substrates (syngaldazine, bilirubin, ascorbic acid, etc.) [1,4,7,9,10]. Placed in direct contact with the electrode material these enzymes can harvest electrons from the electrode, and transfer them through their T1 site to the T2/T3 copper center, at which the oxygen reduction reaction (ORR) takes place [10–14]. Thus, MCOs are extremely appropriate for the development of enzymatic electrodes utilizing direct electron transfer (DET), i.e., direct communication between the enzyme active site and the electrode surface. These types of electrodes have been gaining attention, especially in the past several years. DET-based electrodes do not require the use of dissolved mediators to carry out electron passage from the electrode to the enzyme or vice versa [9,10,15] and they exhibit redox potentials close to the redox potential of the enzyme itself [10]. For multi-copper oxidases the redox potential of oxygen reduction depends on the reduction potential of the T1 site and can vary from 0.23 to 0.59 V (vs. Ag/AgCl) based on the type of the enzyme, its source and pH [9,10]. For bilirubin oxidase (BOx) the redox potential of T1 was determined to be 0.26 V (vs. Ag/AgCl) at pH 5.3 and 0.44 V at pH 7.4 (vs. Ag/AgCl) [10,15,16]. In our previous investigations we determined the formal redox potential of the BOx T1 copper center to be 0.55 V (vs. Ag/AgCl) in 0.1 M phosphate buffer at pH 6.8 [10]. A gas-diffusional cathode assembled with this enzyme and constructed slightly differently from the previously utilized BOx electrode herein described exhibited an open circuit potential of 0.55 V (vs. Ag/AgCl) in 0.1 M phosphate buffer, pH 7 [15].

The prevailing part of developed biocathodes reduce dissolved oxygen, from aqueous phase, rendering them impractical for bio-fuel cell applications due to low oxygen concentration and the need for external, power consuming oxygen supply [3,17,18]. The majority of these cathodes exhibit current densities in the range of 0.05–0.5 mA cm⁻² with the latter achieved after major improvements [5]. At the same time simplistic GDAB enzymatic cathodes are generating current densities in the range of 0.3–2.0 mA cm⁻² [6,7,9,15,19]. Kontani et al. developed GDAB cathode that is capable of achieving current densities of 2.7 mA cm⁻² at 0.00 V (vs. Ag/AgCl) [4]. The same cathode immersed in buffer solution with dissolved O₂ generates current densities from 4.5 to 9 times lower in comparison to the air-breathing mode, irrefutably showing the advantages of GDAB. With precise control of hydrophobicity of the cathode this group managed to attain current densities as high as 20 mA cm⁻² – the highest reported in the literature for biocathodes. The main drawback of this work is the utilization of CueO, which reduces oxygen with large overpotential due to lower formal redox potential of the T1 center (0.38–0.40 V vs. Ag/AgCl) [20]. Similar current values have been reached only by mediated oxygen reduction of dissolved O₂ [18,21]. Gallaway and Calabrese Barton managed to develop laccase cathode with superior performance by utilizing of osmium-based redox polymer mediator [18]. Laccase-based “floating” air diffusion cathode was constructed by Shleev and his group [6]. Achieved current densities were in the range of 0.3 mA cm⁻² in air and 0.6 mA cm⁻² in pure oxygen. Observed enhancement in the cathode’s performance is obviously due to the increase in oxygen concentration. They also noticed that the immobilization of the enzyme on carbon BM-4 leads to an increase of the enzyme stability in comparison to the enzyme in homogeneous solution. It should be mentioned that our research group has a leading role in the development of GDAB biocathodes based on MCO utilizing DET. GDAB electrodes using laccase [7,9,22] and bilirubin oxidase [15,19] were developed and successfully implemented in enzymatic [22] and even in microbial biofuel cells [23].

1.1. Gas-diffusional air-breathing electrodes

All common GDAB electrodes share several requirements: i) high porosity and gaseous oxygen permeability; ii) prevention of electrolyte leakage and iii) electrical conductivity [7,24]. Typical air-breathing electrodes consist of two layers: a porous, hydrophobic, electrically conductive gas-diffusion layer (GDL) and a catalytic layer (CL) containing the enzyme. The GDL is exposed to the air while the CL is situated facing the electrolyte [24]. For effective oxygen transport, the porosity of the hydrophobic GDL must be high (~ 0.9 cm² g⁻¹), providing channels for fast oxygen diffusion [7,24]. The hydrophilic CL should be susceptible to electrostatic or chemical interactions with the enzyme [7]. The enzyme, immobilized at the electrode surface, is kept in constant contact with the liquid electrolyte, which preserves its natural functions and provides an efficient proton transfer. Usually the rate of the enzymatic oxygen reduction is limited by diffusion [4,15]. The diffusion of air through the GDL occurs at a specific rate [24]. Once atmospheric oxygen reaches the CL its diffusion rate is greatly attenuated due to the decrease in the available hydrophobic zones. The electrochemical reduction of oxygen occurs at the three-phase boundary: oxygen–catalyst–electrolyte or gas–solid–liquid [24] due to the presence of partially flooded zones in the catalytic layer. Changes in the thickness, porosity and hydrophobicity of the GDL as well as the ratio of catalyst/hydrophobic material of the CL can greatly affect the properties of the electrode [24].

1.2. Statistical approaches for experimental design and data processing

1.2.1. Design of experiments (DOE)

Changing variables one factor at a time—the typical approach researchers employ based on a trial-and-error method—is a lengthy process for incremental system improvement and understanding. This technique, though very informative, is extremely time-consuming. DOE, a computer-enhanced, systematic scheme that considers all factors simultaneously, is a more effective approach [25,26].

DOE provides a structured and organized approach of conducting and analyzing control experiments to evaluate the factors that are affecting the system being tested [26]. The input variables for the system/experiment are called *factors* and the measurands of the experiment, which characterize the system performance, are called *responses* [26]. In a two-level DOE two values for each factor, called levels, are used. These levels should be spaced such that the difference in the response can be detected, but the levels should not be outside the working range. The use of only two levels in the DOE implies that the analysis is based on an assumption of linear and monotonic change in the response variable [26]. Each combination of the high/low levels for individual factors is called a *treatment* and the number of tests for each treatment is called the *replicates* [27].

DOE fits the final responses into mathematical equations. On the basis of those equations models are subsequently developed that can predict what the response of any given combination of parameters’ values would be [25]. These predictive models provide a way for system improvement by finding the best combination of factor values within the test set [26].

1.2.2. Principal component analysis (PCA)

PCA is a multivariate analysis method, commonly used to reduce the dimensionality of large data sets [28–30]. It is the most popular statistical technique for finding patterns in data of high dimension and expressing the data in such a way as to highlight their similarities and differences [28,29]. PCA facilitates the visualization of the variables responsible for correlations and anticorrelations

among samples [31]. The data in PCA are arranged in matrices, wherein the rows correspond to samples and the columns correspond to variables. On the basis of singular value decomposition, the original data matrix is transformed into a set of new variables, called *loadings*. Thus, PCA transforms a number of correlated or possibly correlated variables into a number of uncorrelated ones, called *principal components* [32]. The first principal component has the largest possible variance and the second, orthogonal to the first, has the largest possible inertia [30].

PCA has been successfully applied in different areas, such as processing X-ray photoelectron spectroscopic data, clustering gene expression data, studying inversion of temperature distribution, correlating limits on grain yield with soil chemicals and physical attributes, face recognition systems, etc. [33–38].

The aim of this study is to design and improve a GDAB cathode based on enzymatic oxygen reduction performed by BOx. For that purpose two statistical approaches were used: DOE and PCA. DOE was used to identify the factors and their levels that would lead to cathode enhancement, after which PCA was applied to confirm or reject the conclusions made by DOE and to visualize the relative significance of each contributing factor in the final performance. The benefits of statistical approaches for experimental design and data processing, which are well known and widely utilized in analytical chemistry, are shown also in this type of material design and optimization research.

2. Experimental

2.1. Materials

BOx (from *Myrothecium verrucaria*) was purchased from Amano Enzyme Inc. (Nagoya, Japan). Potassium dibasic and monobasic phosphates were used to prepare the electrolyte solutions and were purchased from EMD Chemicals Inc. (Darmstadt, Germany). Potassium chloride obtained from MacronTM Chemicals (Phillipsburg, USA) was added as an indifferent electrolyte. Absolute isopropyl alcohol (IPA) 99.99% was from Honeywell Burdick & Jackson (Muskegon, USA). WhatmanTM (Maidstone, UK) qualitative cellulose filter paper, Grade 2, was used for the construction of the paper-based fuel cell (PBFC). Transparent vinyl cover tape with

hydrophilic adhesive (G&L Precision Die Cutting, Inc., San Jose, CA) was used to hold the electrodes.

2.2. Cathode preparation

The designed cathode consisted of TG-H-060 Toray paper (Fuel Cell Earth LLC), a layer of teflonized carbon black (XC72R with 35 wt % PTFE) and highly conductive multi-walled carbon nanotube paper (Buckeye paper, Grade CMN, Buckeye Composites Kettering, OH). The Toray paper (TP) was cut in precise shape using a computer-controlled x–y knife plotter (Graphtec FC7000-75, Western Graphtec Inc., Irvine, CA) [19]. The circular part of the TP served as mechanical support for the catalytic layer while the rectangular extension was used to connect the circuitry. The outer side of the TP was in direct contact with air and the inner side was covered with the teflonized carbon black. The procedure for the hydrophobized carbon black preparation, designated here as XC35, was previously described [7]. The outer side of XC35 served as a gas-diffusion layer whereas the inner side was pretreated with IPA to achieve a gradient from hydrophobic to hydrophilic properties across the layer. The Toray paper, the XC35 layer and the Buckeye paper were fused together by hydraulic pressing. The Buckeye paper was used to support the catalytic layer. It possessed higher surface area and lower hydrophobicity than the teflonized carbon black.

A solution of BOx in 0.1 M phosphate buffer (PBS), pH 7.5 was immobilized on the Buckeye paper by physical adsorption. When the enzyme was immobilized for 24 h the enzyme solution was dropped at the electrode surface and kept at 4 °C; otherwise it was dried with N₂ immediately after deposition.

2.3. Electrochemical characteristics

Electrochemical cell, utilized in this study (Fig. 1), is designed around microfluidic passive flow system, based on capillary action, which provides a steady flow of electrolyte [39]. Filter paper was used as main structural element. It served as fuel delivery system and a separator, as well as proving mechanical stability. The specific fan shape of the paper maintained a constant volumetric flow through the reaction zone, where the enzyme was deposited. The fabricated device is cheap, versatile and biodegradable, which

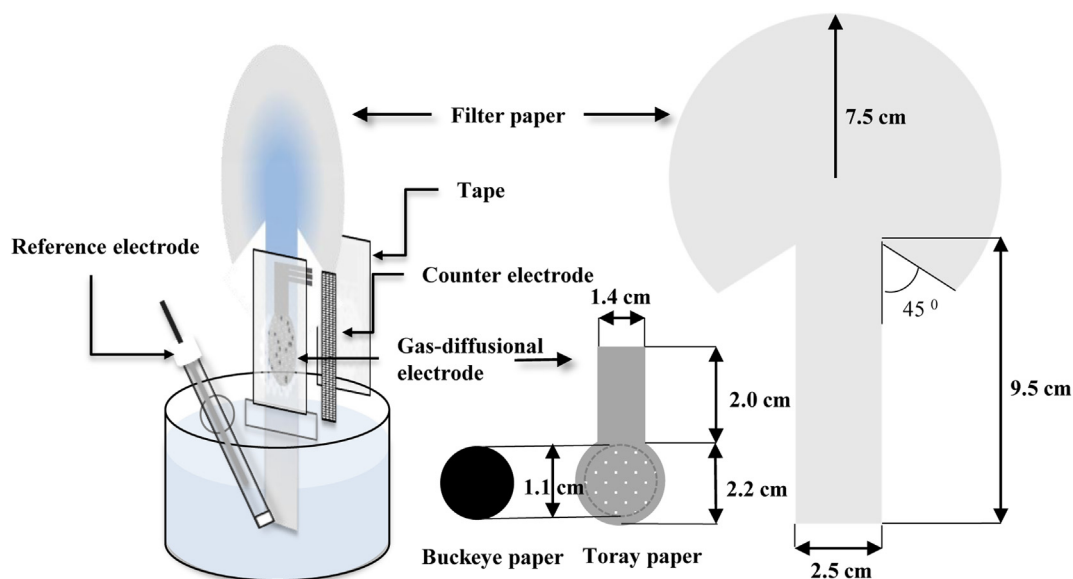


Fig. 1. Schematic representation of the paper-based biofuel cell used for the electrochemical measurements.

Table 1
Main factors, determining the cathode performance and their levels.

Factor	Low level (−1)	High level (+1)
Hydraulic Pressure (psi)	500	800
Pressing time (min)	1	5
Amount of BOx (mg)	5	10
Volume of IPA (μL)	100	300
Volume of phosphate buffer (μL)	100	300
Amount of XC35 (mg)	50	150
Time of BOx adsorption (h)	0	24

makes it very suitable for the construction of biofuel cells. This paper-based fuel cell (PBFC) has been successfully applied in our previous studies of BOx cathodes [19].

Whatman® Grade 2 filter paper was cut in the shape of a fan (Fig. 1). The tested cathode (working electrode) and a platinum mesh (counter electrode) were assembled on opposite sides of the filter paper. The electrodes were sealed to the paper by a transparent vinyl cover tape. It provided mechanical stability and better contact between the electrodes and the paper, while lowering the evaporation rate in the region containing the enzyme.

The foot of the filter paper was soaked in PBS (pH 7.5). The electrolyte flowed up through the length of the paper driven by capillary force and evaporated when it reached the fan. This established a constant wicking speed throughout the filter paper. A 3.0 M Ag/AgCl reference electrode was used to measure and control the potential of the working electrode.

2.4. Design of experiments

A Plackett–Burman eight-run experimental design (QI Macros, DOE Software for Excel) was used to create the sets of experiments to enhance the cathode's operational characteristics and to establish the main parameters influencing the electrochemical behavior of the constructed gas-diffusional cathode. The number of treatments was eight and the number of replicates for each treatment was three.

2.5. Principal component analysis

PLS_Toolbox 5.0 for Matlab was used for the multivariate analysis of the data [33]. PCA, using autoscaling as a preprocessing option (mean centering and scaling to unit variance), was the default method of data analysis.

3. Results and discussion

3.1. Design of experiments

A gas-diffusional air-breathing cathode with BOx was designed and then improved by DOE. The eight-run experimental design was

used to demonstrate the relative significance of the components comprising the gas-diffusional cathode and to enhance its performance. The first step in the Plackett–Burman design is defining the main factors determining the system behavior and the high/low values for the levels to be varied. For the BOx cathode studied (See Experimental section) seven factors were considered as basic factors influencing its performance (Table 1). Each of these factors was varied and tested at high (+1) and low (−1) levels. Thus a set of cathodes/treatments with the elements, called factors, and their magnitudes/levels, given in Table 2, were constructed and tested. The cathode design is represented in Fig. 2.

For the evaluation of the designed cathode performance polarization curves were taken (Fig. 3) and, as an estimator of their operation, current densities at 0.30 V (vs. Ag/AgCl) were chosen. The 0.30 V potential was chosen as a result of statistical analysis of the obtained data. Recorded current densities for multiple electrodes had higher reproducibility (lowest standard deviation) at this potential as compared to all other applied potentials from polarization curve (data not shown). In terms of statistical comparison of data, it is highly recommended and more reliable to compare data with lowest uncertainties. All treatments were performed in triplicate and the average value was taken as final response.

The basic factors (Table 1) can be separated into two main groups, by their influence on the electrode characteristics and performance. The first group contains the amount of BOx, the volume of phosphate buffer and the time of BOx adsorption. The biggest impact of these factors is observed mainly at lower current densities, at which activation losses dominate. The second group includes hydraulic pressure, pressing time, amount of IPA and amount of XC35. It is believed that these factors determine the cathode electrochemical response at higher current densities, where diffusional limitations occur. The dependences between the factors are represented in Fig. 4. Fig. 4 illustrates observed factor interactions in two ways: linearly (graph inserts) and through the use of a bar graph. The linear representation displays information about the presence/absence of dependence between the factors. Factors that are independent of one another are represented by parallel lines on the graph, indicating that changing the value of one factor would not have any effect on the other factor. However, the presence of an intercept, as in Fig. 4B and C, means that the two factors are interdependent and changing the value of one factor would directly affect the second factor. Bar graph representation allows for simplicity in data interpretation: if 1 bar is higher than the other, than it can be easily determined which value (high/low) gives the best result, in this study – the higher current.

The main factor of the first group is the enzyme amount. When the cathode operates at low current densities—in the region where transport is not limiting—the current generated from the air-breathing cathode increases with increasing thickness of the

Table 2
Different combinations of the factors and their levels for the set of cathodes prepared and tested during the study.

Factor design	Hydraulic pressure	Pressing time	Amount of BOx	Volume of IPA	Volume of PB	Amount of XC35	Ads. time ^a	Response, $\mu\text{A cm}^{-2}$ (average)	SD ^b	Samples, $\mu\text{A cm}^{-2}$		
										1	2	3
Cathode#1	+	−	−	+	−	+	+	218	18	200	218	236
Cathode#2	+	+	−	−	+	−	+	195	17	195	212	178
Cathode#3	+	+	+	−	−	+	−	273	17	277	254	288
Cathode#4	−	+	+	+	−	−	+	254	17	258	236	269
Cathode#5	+	−	+	+	+	−	−	310	24	310	334	287
Cathode#6	−	+	−	+	+	+	−	377	20	382	355	395
Cathode#7	−	−	+	−	+	+	+	411	16	405	429	400
Cathode#8	−	−	−	−	−	−	−	164	19	167	144	181

^a Time of BOx adsorption.

^b Response standard deviation in $\mu\text{A cm}^{-2}$.

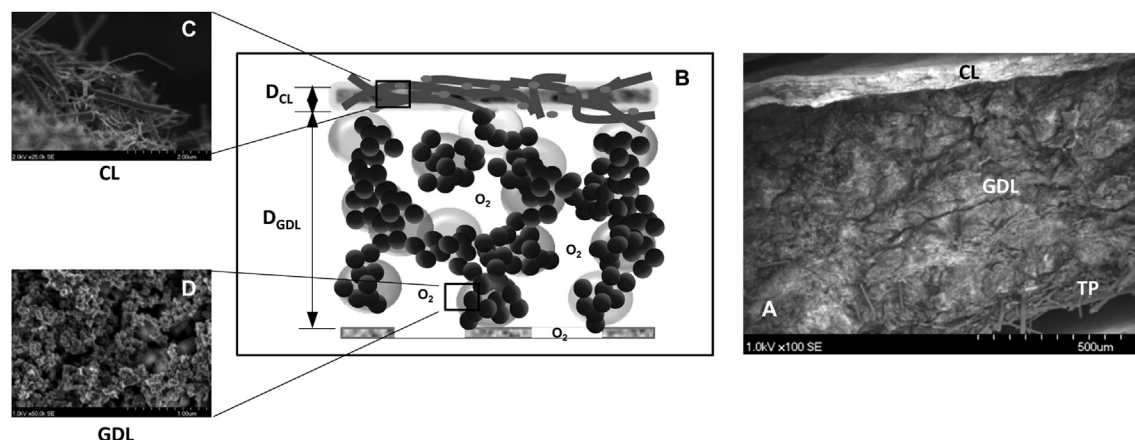


Fig. 2. A) SEM image of cross-section of the improved GDAB cathode; B) Schematic illustration of the cathode layers; C) Higher magnification SEM image of the catalytic layer (CL); D) Higher magnification SEM image of the gas-diffusional layer (GDL).

catalytic layer or amount of the catalyst [24]. Taking into account that the enzyme is catalyzing the reduction of oxygen to water, it is expected that the current densities would be proportional to the enzyme present at the electrode surface. Fig. 4A clearly shows that the value of the recorded current increased with increase in the amount of BOx deposited onto the electrode. Based on these results it can be affirmed that the amount of BOx at its high level is preferred for electrode fabrication. The higher volume of buffer keeps the enzyme wet during adsorption and thus prevents its denaturation and loss of activity.

When the enzyme amount and the time allowed for its adsorption were increased, the enhancement of the current density was notable (Fig. 4B). Therefore, the improved procedure for the cathode preparation included 10 mg BOx physically adsorbed for 24 h.

At higher current densities, due to increased transport limitations, the generated current densities strongly depend on the diffusion of oxygen [24]. Thus the pore size and hydrophobicity of GDL considerably influence the overall electrode performance. The magnitude of the applied hydraulic pressure and the time of its application determine the pore size of GDL. The amount of XC35

defines the thickness, while the volume of IPA determines the hydrophobic/hydrophilic gradient of the GDL. For this reason the ratio between teflonized carbon and IPA volume is an important criterion influencing the electrode performance. In our previous investigations we found that 35% PTFE gives sufficient hydrophobic character to the layer, without affecting the conductivity of the carbon material [7].

According to Fig. 4C, higher current densities are observed when the hydraulic pressure applied to fuse the layers together is at the low level and application time is 5 min. This can be explained as formation of a homogenous GDL with larger-sized hydrophobic pores. At the same time, using high amount of XC35 (Fig. 4D) gives a thicker hydrophobic layer, which increases the amount of oxygen available to the catalyst. Barton et al. showed that the magnitude of the current generated from a laccase cathode varies with the thickness of the carbon support layer, and a thicker layer leads to higher current densities [21].

The ideal GDL possesses an increasing hydrophilic character towards the electrolyte. The ratio between the hydrophobic and hydrophilic character of the carbon material determines the performance of gas-diffusional electrodes [7]. The last factor from the second group is the volume of IPA. The purpose of adding IPA is to create a gradient in the GDL from hydrophobic to hydrophilic properties—hydrophobic at the outer and hydrophilic at the inner side, which is in contact with the catalytic layer. At high volumes of IPA the gas-diffusional part of the cathode would no longer be hydrophobic and diffusion of oxygen through it would be hindered, thus the generated current densities would be due to reduction of dissolved oxygen in the electrolyte, not of oxygen in the air. Therefore the volume of IPA added should be low at high amounts of XC35 (Fig. 4E).

In summary, the cathode with improved performance should be prepared as follows: Toray paper, 150 mg XC35 with 100 μL IPA and the Buckeye paper are fused together by hydraulic pressure of 500 psi, applied for 5 min. BOx (10 mg) is dissolved in 300 μL of 0.1 M phosphate buffer, pH 7.5, and drop-cast on the surface of the nanotube paper. The electrode is then kept for 24 h at 4 $^{\circ}\text{C}$. To confirm this statement with real experimental data, a cathode with the specified values of the factors was constructed and tested. The observed polarization curve is shown in Fig. 3.

The current density generated from the improved electrode ($755 \pm 39 \mu\text{A cm}^{-2}$ at 0.3 V vs. Ag/AgCl) is two times higher than the current density observed with the previous best-performing cathode (cathode #7) and five times higher than the worst-performing cathode (cathode #8). Based on these results it can be concluded

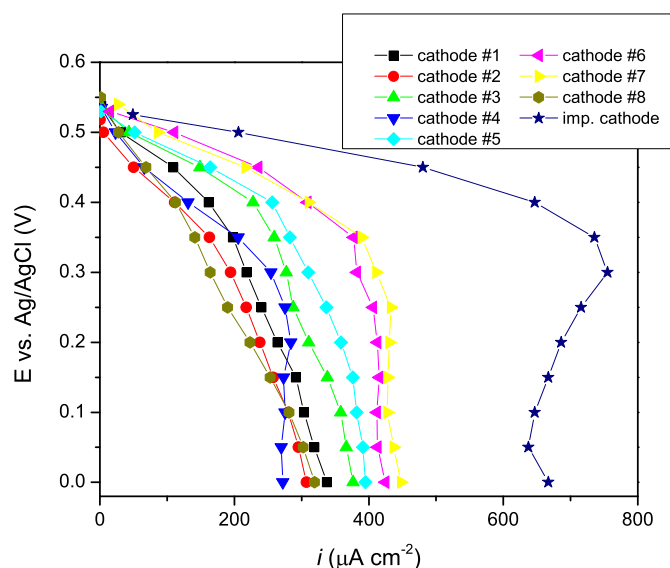


Fig. 3. Representative polarization curves of the cathodes described in Table 2 and the improved cathode, designed based on DOE.

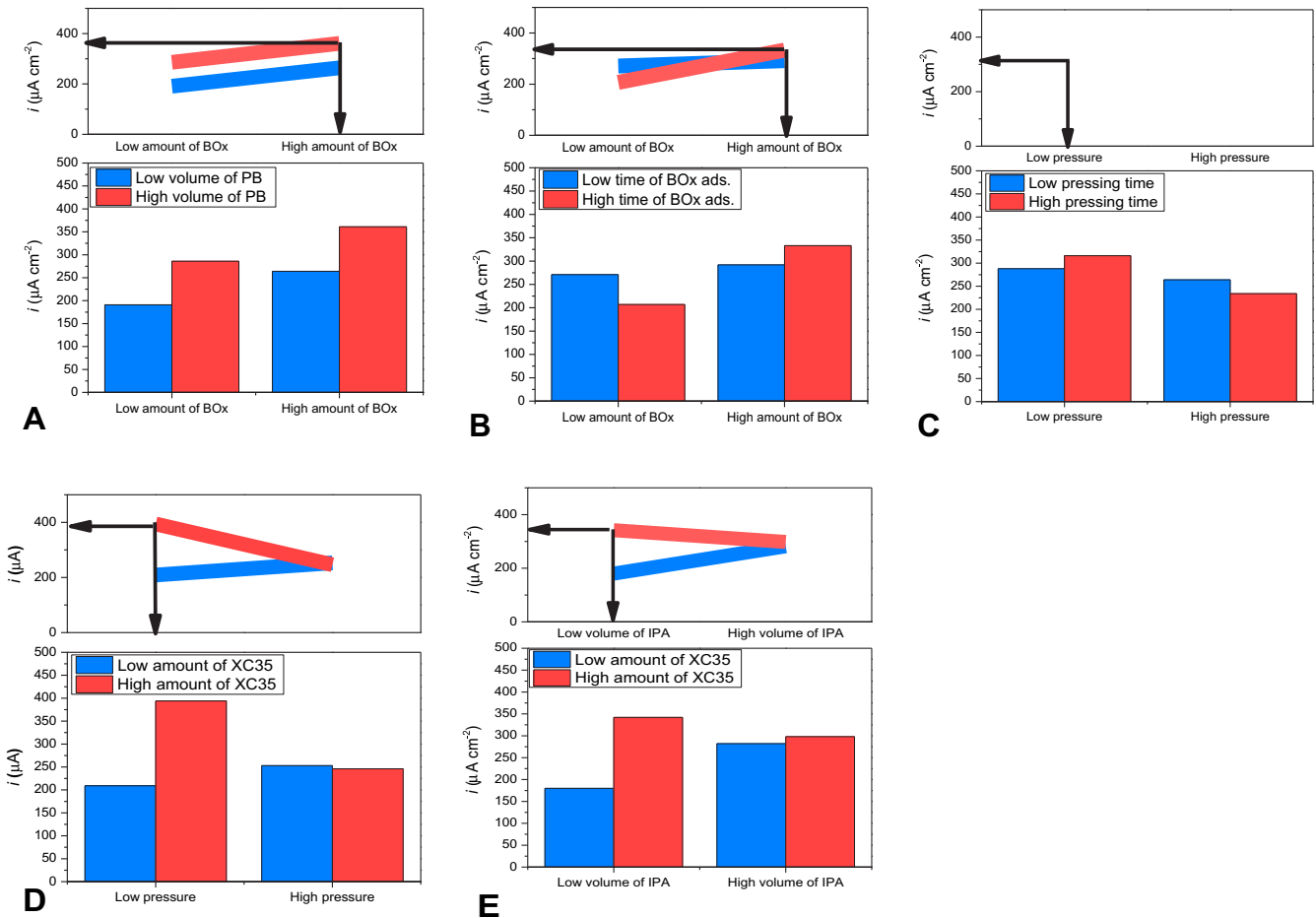


Fig. 4. Dependences between the main factors: A) pressure/pressing time; B) pressure/amount of XC35; C) amount of XC35 and IPA; D) enzyme amount and the volume of phosphate buffer; E) BOx amount and the time of its adsorption. The figure represents the dependence of the cathodes response (current densities $\mu\text{A cm}^{-2}$) from the factor levels.

that the approach utilized for the electrode enhancement led to notable improvement in the electrode's operation.

To further understand which factors have major impacts on the cathode performance, the effects of the individual factors, called *main effects*, were estimated. For a balanced design, a simple way to calculate the main effect of a factor is to calculate the difference of the mean values of the response at its high and low setting [27]. Applying this approach gives the following arrangement of factors as percentages (Fig. 5):

Three main factors exerting approximately equal effects on the cathode's performance are: volume of phosphate buffer, amount of teflonized carbon black and amount of enzyme. The pressing time and the time of adsorption are considered insignificant. It should be

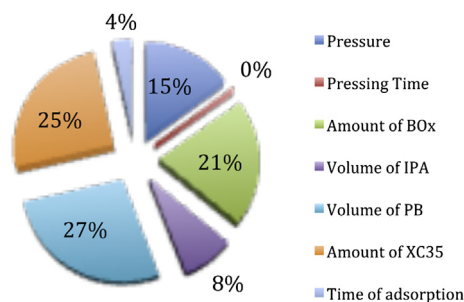


Fig. 5. Shares of the main effects determining the operational characteristics of the designed air-breathing cathode.

noted that the main effects of the factors were estimated by fractional factorial design, which is not as accurate as the full factorial design [27].

Two of the factors with largest impact are connected with the construction of the catalytic layer and the cathode operation at low current densities. In this region the generated current can be represented with the following equation:

$$i = \ln S \cdot \vec{k} (k_0 \cdot P_0) \cdot \exp\left(\frac{azF}{RT} E\right) \quad (1)$$

where S is the wetted surface of the catalyst, \vec{k} is the rate constant of the electrochemical reaction, k_0 is the solubility and P_0 is the partial pressure of oxygen. If the same expression is represented in Tafel form it becomes:

$$E = \frac{RT}{azF} \ln S \cdot \vec{k} (k_0 \cdot P_0) - \frac{RT}{azF} \ln i \quad (2)$$

where E is the applied potential and the plot of E vs. $\ln(i)$ is linear, having an intercept a with the abscissa:

$$a = \ln S \cdot \vec{k} (k_0 \cdot P_0) \quad (3)$$

a is a parameter characterizing the electrode activity [24]. A higher values of a corresponds to higher cathode activity. Basically, the parameter a is equivalent to $\ln(i_0)$ [40].

Using this concept the activity of the cathodes was evaluated and compared to the activity of the improved cathode to estimate the effectiveness of the DOE as applied. The results for the activity and the parameters affecting it are represented in Table 3. As evident from the table, two electrodes show distinctly higher activity—cathode #7 and the improved cathode. Both have 10 mg BOx, dissolved in 300 μL of buffer and adsorbed for 24 h. These results serve as confirmation that enzyme loading and incubation time are important parameters for the cathode activity and that the improvement procedure led to significant enhancement of the electrode's performance at low current densities. In order to demonstrate the significance of the improvement in response, the current density of cathode #7 ($411 \pm 16 \mu\text{A cm}^{-2}$) and the improved cathode ($755 \pm 39 \mu\text{A cm}^{-2}$) was compared using Student's *t*-test. The assumption: "the two means of the data sets are identical", was taken as a null hypothesis (H_0). The alternative hypothesis (H_1) is: "The means are significantly different". When the calculated *t*-value ($t_{\text{calc.}} = 8.152, N = 2$) was compared to the theoretical *t*-value ($t_{\text{theor.}} = 6.956, N = 2$) it was determined that $t_{\text{calc.}}$ had lower value than $t_{\text{theor.}}$, for *p*-value of 0.01 or 99% confidential interval. The smaller $t_{\text{calc.}}$ than the $t_{\text{theor.}}$ means that the H_0 must be rejected, while H_1 becomes the only acceptable hypothesis. Therefore, the two means (current densities) are statistically different, which confirms previous observation about achievable current densities for optimized and non-optimized (#7) cathodes.

Generally, the performance of the air-breathing gas-diffusional cathodes depends not only on the activity of the catalyst/enzyme but also on the transport limitations of oxygen through the porous structure of the electrodes [24,41]. This is clearly visible at high current densities, where transport losses take place. Thus, comparing the results at 0.00 V (vs. Ag/AgCl), the current density generated by the improved cathode is $670 \pm 98 \mu\text{A cm}^{-2}$ versus $436 \pm 49 \mu\text{A cm}^{-2}$ by cathode #7 and $269 \pm 31 \mu\text{A cm}^{-2}$ by cathode #4, the best- and the worst-performing cathodes, respectively, in the region of low potential/high current density. Evidently the enhancement procedure led to an increase not only of the electrode activity at low current densities, but also of oxygen diffusion through the GDL, i.e., the gas-diffusional properties of the cathode were improved.

3.2. Principal component analysis

In order to verify the conclusions made by the DOE we processed the same data using principal component analysis. PCA was chosen as an independent method of data analysis because multiple contributing factors are associated with the system response. The variables used in the data matrix for the PCA are identical to the factors in the DOE. The generated current density at 0.30 V (vs. Ag/AgCl) is used as a measure of the cathode's operational efficiency.

PCA extracts mathematical principal components (PC) from linear combination of original variables. The first PC accounts for

Table 3
Activity of the cathodes and the parameters influencing this activity.

Cathode#	Activity (μA)	BOx amount (mg)	PB (μL)	Time of adsorption (h)
1	0.20	5	100	24
2	0.01	5	300	24
3	0.45	10	100	0
4	0.25	10	100	24
5	0.08	10	300	0
6	0.29	5	300	0
7	1.38	10	300	24
8	0.03	5	100	0
Improved cathode	1.52	10	300	24

Bold indicates the cathodes with the highest activity due to the combination of 10 mg BOx, 300 μL of PB and 24 hours of adsorption.

the largest part of the variance in the data, the second PC accounts for the second-largest part of the variance, and so forth. The results of PCA herein will be displayed as loading plots, showing contributions from original variables, and biplots, showing both samples and variables for two principal components. The dataset was auto scaled so all of the variables would be equally weighted and components with small magnitudes would carry the same importance as those with larger values in the PCA data matrix analysis.

Estimation of the loading factors, or *variables*, shows the same trend as was observed for the factor effects with DOE (Fig. 6). The first PC captures $\sim 30\%$ of variance in the data and separates well performing samples from poor performing samples. The pressing time, amount of BOx, volume of buffer and amount of XC35 all have positive loadings, which mean that when they increase the response also increases. The other two factors: hydraulic pressure and volume of IPA have negative loadings and their increase leads to a decrease in the final response, i.e., the generated current densities. Thus the influence of these factors, as determined by PCA is exactly the same as their influence as determined by the DOE method. According to the loadings, the factors with smaller influence on the cathode's performance are the time of adsorption, volume of IPA and pressing time. In contrast with DOE, where these factors were considered insignificant to the overall performance of the cathode, PCA shows that they do have substantial positive (pressing time and time of adsorption) and negative (volume of IPA) contributions to the cathode's response.

Fig. 7 shows a biplot, which is a display of the contribution of the scores (of the tested cathodes) and the variables to the first and second principal components. The first PC captures 30% while the second PC captures 12% of the variance in the original data. The aspect ratio of the biplot is kept to represent the % variance of both PCs represent. The first principal component contrasted the improved cathode and cathode #7, which yielded the highest current densities, with cathodes #1, 2 and 8, which generated the lowest current densities. The only difference between preparation of cathode #7 and the improved cathode is the magnitude of the pressing time. According to the DOE's conclusions, the pressing time is an insignificant factor and should not measurably influence the value of the final response. As evident from Fig. 3, the current density, generated from the improved electrode is twice the current density of cathode #7. Therefore the conclusions of PCA are in better agreement with the experimental observations. As previously mentioned the main effects of the factors in DOE were estimated from a fractional factorial design, which, in contrast to the full factorial design, is not as accurate.

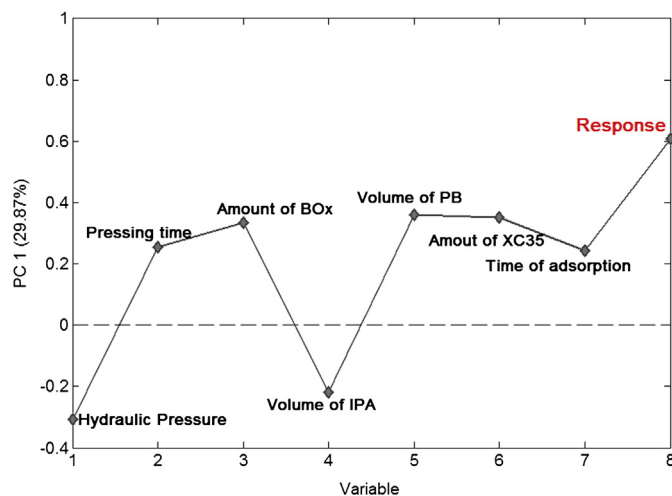


Fig. 6. Variables/loadings plot to first principal component (PC1).

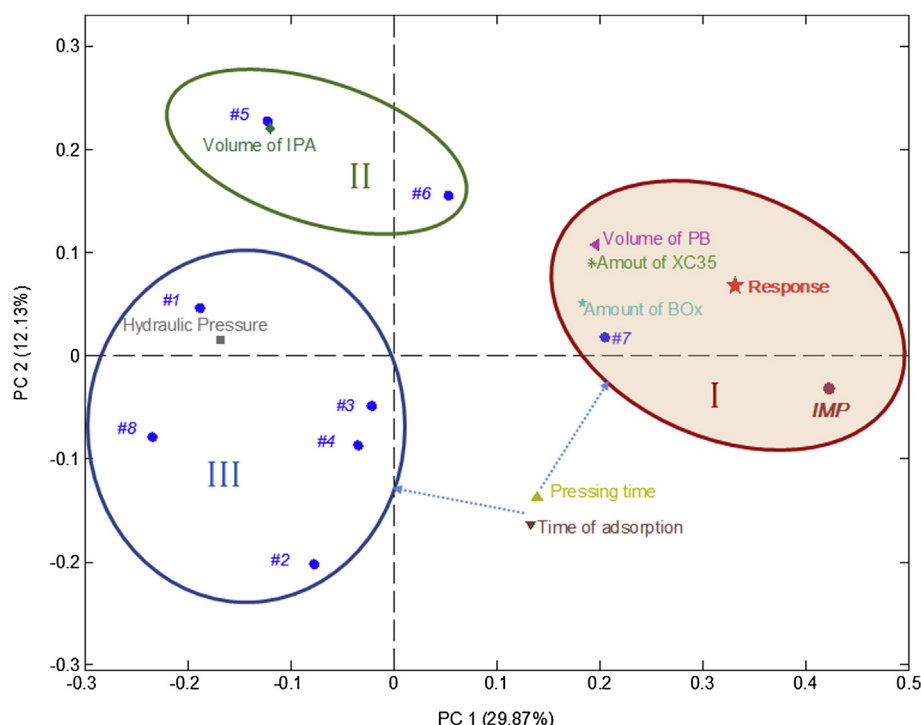


Fig. 7. PCA cathode characteristics. Loadings of the variables plotted on the first two components. The improved cathode is marked as IMP.

Principal component 1 (PC1) separated the variables with positive effects and those with negative effects on the cathode response. A positive effect is an increase in response value with an increase of the variable's magnitude, while a negative effect is a decreased response with an increase of the variable's magnitude. On the biplot, the variables located in close proximity to the response—i.e., the factors with the biggest impact on the response value—are the amount of BOx, the volume of IPA and the amount of XC35 (Fig. 7). This coincides with the conclusion made by DOE. The biplot also shows that in PC1 the volume of IPA and the hydraulic pressure have negative contributions, being opposite to all other factors in their effect on the response.

Principal component 2 (PC 2), capturing 12% of variance, separates samples by added volume of IPA versus pressing and adsorption time.

A separation of variables and cathodes into three groups is depicted in the biplot in Fig. 7. Group I contains the cathodes with the highest performance due to amount of buffer, amount of XC35 and amount of BOx. The second group (II) separates those cathodes that have largest levels of IPA. Remaining cathodes are in group III for which hydraulic pressure was largest and response was lowest, particularly for cathode #8. The pressing time and the time of adsorption are located between groups I and III as these factors have equal contribution to the best and worst samples. As we have discussed, the magnitude of pressing time, the only difference between the improved cathode and cathode #7, doubled the performance of the improved cathode. However, the PC1 biplot shows that this factor by itself, without the synergistic effect of the amount of buffer, XC35 and enzyme, does not result in better performance.

4. Conclusions

Two different statistical methodologies, DOE and PCA, were employed to investigate and improve the performance of an air-breathing gas-diffusional enzymatic cathode. Design of experiments

was used as a structured and organized method of conducting and analyzing controlled tests to evaluate the factors affecting the performance of the tested system. Principal component analysis was used to confirm the conclusions made by DOE and to show synergistic effects of factors essential for best performance.

Based on DOE methodology, the set of optimal factors was predicted and an improved cathode was fabricated. The current densities generated by this cathode were higher at all potentials in comparison with all other cathodes constructed and tested during the study. It was established that three major factors contributed to higher cathode performance: the amount of enzyme, the volume of phosphate buffer used for enzyme immobilization, and the amount of XC35, which determines the thickness of the GDL. All these factors must be at their high level: (10 mg BOx, 300 μ L PB and 150 mg XC35) for higher cathode performance. The factors with negative effects on the electrode's response were hydraulic pressure and volume of IPA. Thus the improved cathode was constructed and prepared as follows: 150 mg XC35 and 100 μ L IPA pressed at 500 psi for 5 min; 10 mg BOx dissolved in 300 μ L phosphate buffer, adsorbed for 24 h. The current density observed with the improved cathode ($755 \pm 39 \mu\text{A cm}^{-2}$ at 0.30 V vs. Ag/AgCl) was significantly ($p = 0.01$) higher than the current density generated with any other cathode employed in this study.

Applying PCA to the obtained data led to similar conclusions. Moreover, PCA reported that pressing time in synergy with the three factors suggested by DOE vastly affects the performance.

Hence, both of the proposed techniques can be utilized to analyze data and predict results. These two methods can be employed to gain deeper understanding of the influence the building components have on the given system.

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Glossary

Multi-copper oxidases (MCO)

a group of enzymes (laccase, bilirubin oxidase, ascorbate oxidase, etc.) that can reduce oxygen during the oxidation of their natural substrates (syringaldazine, bilirubin, ascorbic acid, etc.)

Direct electron transfer (DET)

direct electron transfer between the enzyme active site and the electrode surface

Gas-diffusional air-breathing electrodes (GDAB)

electrodes with a conjunction of a solid, liquid and gaseous interface, and a catalyst performing an electrochemical reduction of oxygen between the liquid and the gaseous phase

Gas-diffusional layer (GDL)

hydrophobic, electrically conductive carbonaceous layer, exposed to air

Catalytic layer (CL)

hydrophilic, electrically conductive layer containing the catalyst, exposed to the electrolyte

Design of experiments (DOE)

a structured and organized approach of conducting and analyzing control experiments to evaluate the factors that are affecting the system being tested

Principal component analysis (PCA)

a multivariate analysis method, commonly used to reduce the dimensionality of large data sets

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